Heterogeneity of phenotypic characteristics of the modern and ancestral Beijing strains of *Mycobacterium tuberculosis*

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Summary

Background: **The Beijing strain of *Mycobacterium tuberculosis* (MTB) is of great concern because this hypervirulent strain has caused numerous tuberculosis outbreaks. However, the mechanisms that allow the MTB Beijing strain to be highly pathogenic remain unclear and previous studies have revealed heterogeneity within this family.**

Objective: **To determine the association between some phenotypic characteristics and phylogroups of the Beijing strain of MTB.**

Methods: **Eight Beijing strains, 5 modern and 3 ancestral sublineages, were selected from the phylogroups of MTB. The selection was based on copy number of IS*6110* at NTF, region of differences, and single nucleotide polymorphisms. The abilities of these strains to grow intracellularly in THP-1 macrophages, to induce apoptosis, necrosis, and cytokines production were examined using quantitative real-time PCR and commercially available ELISA kits, respectively.**

Results: **There were some significant differences between the two sublineages of the Beijing strain of MTB. The ancestral Beijing sublineages showed higher intracellular growth rates ($p < 0.05$) and necrosis induction rates ($p < 0.01$) than the modern Beijing sublineages. By contrast, the modern Beijing sublineages induced lower apoptosis and protective cytokine responses, i.e., TNF-α ($p < 0.05$) and IL-6 ($p < 0.01$) and higher non-protective IL-10 response. The modern Beijing sublineages may have evolved so that they have greater ability to diminish host defense mechanisms. The slower growth rate and reduced necrosis induction in host cells might allow the bacteria to cause a persistent infection.**

Conclusion: **The results revealed a phylogroup-associated heterogeneity of phenotypes among MTB Beijing sublineages. *(Asian Pac J Allergy Immunol 2014;32:124-32)***

Key words: *Beijing sublineages, Intracellular growth, Cytokines, Apoptosis, Necrosis*

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Introduction

The *Mycobacterium tuberculosis* (MTB) Beijing strain is a highly virulent genotype and has caused many tuberculosis (TB) outbreaks worldwide.[1] Additionally, this genotype is associated with several virulence-related properties, such as drug-resistance;[2] treatment failure; extrapulmonary TB[3] and tuberculous meningitis (TBM).[4] The MTB Beijing strain may be an escape variant caused by

BCG vaccination.[5] This genotype of MTB also causes rapid death with more severe pathological outcomes compared to other genotypes.[6] The Beijing strain shows a higher intracellular growth rate in infected human macrophages[7] and induces less protective cytokine production.[8] However, the virulence-determining factor(s) or mechanism(s) of the Beijing strain remain elusive, although phenolic glycolipids have been identified as one mechanism.[6]

The MTB Beijing strain contains many sublineages that are identified by a series of genetic markers, e.g., IS*6110*, region of differences (RDs), and single nucleotide polymorphisms (SNPs). Based on the presence or absence of IS*6110* in the noise transfer function (NTF) region, the Beijing strain can be divided into modern and ancestral sublineages.[9] SNP markers can also be used to differentiate ancestral and modern Beijing sublineages.[10] The evolutionary distance between modern and ancestral Beijing sublineages has been previously demonstrated.[11] However, the effect of evolution on pathogenicity, as defined by these genetic markers, is not understood. Notably, several studies have suggested heterogeneity of phenotypes among the Beijing strains.[12-14] Additionally, it has been suggested that the most recently evolved modern Beijing strains are more virulent, based on phenotypic properties.[11-14] The understanding of the correlation between phylogenetic variations of the MTB Beijing strain and the phenotypic properties of these bacteria can help us to understand the factors that determine virulence and pathogenicity.

In our previous study, the Beijing strain was the most predominant genotype causing TBM.[4] However, when we compared the phenotypic characteristics (e.g., growth rate, apoptosis, and the level of cytokines) between Beijing and non-Beijing strains isolated from the CSF of TBM patients, no significant differences were observed. In contrast to our results, significant differences in virulence between Beijing and non-Beijing strains have been reported in animal models and clinical correlation experiments.[3,5-8] Therefore, in this study, we further analyzed the phylogroup-associated phenotypes among the MTB Beijing sublineages.

Methods

MTB strains and preparation of mycobacterial inoculum

Eight sequence types (STs) of the MTB Beijing family were selected from a total of 12 distinct branches of the phylogenetic tree of Beijing strains from tuberculous meningitis patients (Table 1).[10] These isolates can be divided into 3 ancestral and 5 modern Beijing sublineages based on IS*6110* in the NTF region.[9] All sequence types used were epidemiologically unlinked (unclustered) and pan-susceptible to anti-tuberculous drugs. The MTB H37Rv strain was used as a reference strain. A heat-killed H37Rv strain of MTB (80 °C for 30 min) was used as a control.

The MTB Beijing strains and the H37Rv control were freshly prepared for each of the experiments, which were performed in triplicate, by growing to late-log phase (14 days) in Middlebrook 7H9 (M7H9) broth. After 14 days, all liquid cultures were centrifuged at 4,000 rpm for 8 min, the supernatant was removed, and the pellet was re-suspended in 5 ml of M7H9 medium. The liquid cultures were filtered through a 20-μm filter syringe set and adjusted by measuring the OD at 600 nm of 0.1 (equivalent to $1x10^8$ cells/ml). These filtered suspensions were used as working inoculums and the bacterial concentration was determined by quantitative real-time PCR (qPCR).

Table 1. The sublineages of MTB Beijing strains selected for phenotypic assays.

	MTB Beijing strains	Laboratory number	DST	Clinical data
1	Modern	43-16922 (24)	Pan susceptible	Available
2	Modern	47-6903 (109)	Pan susceptible	Available
3	Modern	CSF 4119 (158)	NA	NA
4	Modern	43-10148 (8)	Pan susceptible	Available
5	Modern	CSF 3394 (110)	Pan susceptible	NA
6	Ancestral	CSF 1796 (80)	Pan susceptible	NA
7	Ancestral	CSF 2865 (89)	Pan susceptible	NA
8	Ancestral	43-06042 (102)	Pan susceptible	Available
9	Control	ATCC27294	NA	NA

DST= drug susceptibility test, NA= data are not available,

Beijing strains were selected from the phylogentic tree of previous studies .[11]

The ancestral and modern Beijing sublineages were mainly divided based on the copy number of IS*6110* in the NTF region.

Determination of bacterial concentration by qPCR

Fifty microliters of each inoculum were added to 150 µl of TE buffer containing 0.1µm glass beads in suspension. The bacterial DNA was extracted by incubating the bacteria at 80°C for 30 min and then physically rupturing them in a Hybaid Ribolyser Cell Disrupter (Hybaid, Ltd., Ashford, UK) at speed 5 for 3 min. The extracted DNA was used as a template for qPCR. The *rpoB* gene was amplified by using primer sequences (5'-3') of *rpoB*-F: CCG CGA TCA AGG AGT TCT TC and *rpoB*-R: GCC GAT CAG ACC GAT GTT G. A 220-bp segment was amplified by PCR by adding 2 µl (5 ng) of DNA template in a final volume of 20 µl (10 µl PerfeCTa^{TM} SYBR Green Super Mix (Quanta Bioscience, Maryland, USA), 20 pmol of primers (10 pmol of each primer in a volume of 0.1 µl) and 7.8 µl purified water). The amplification program consisted of 1 cycle of 3 min at 95ºC, followed by 40 cycles of 10 sec at 95ºC and 30 sec at 65ºC. Various concentrations of an MTB DNA standard ($2x10^1$ - $1x10^7$ copies per 2 µM) were used to generate a standard curve. The copy number of MTB, which corresponded to the threshold cycle (Ct) and to the fluorescence signal, was estimated by comparison with the standard curve. The qPCR assays were performed in triplicate using duplicate samples. Three independent qPCR experiments were performed.

Culture and activation of THP-1 cells

The human monocyte cell line THP-1 was cultured in RPMI1640 supplemented with 2 mM L-glutamine and 10% heat-inactivated fetal bovine serum. Human monocyte cells were seeded at a concentration of 2.5×10^5 cells in a volume of 1 ml per well in 24-well tissue culture plates with 100 nM/L Phorbol 12-Myristate 13-Acetate (PMA) and allowed to adhere and differentiate to macrophages at 37°C in 5% CO_2 for 20 hr. After 24 hr of activation with PMA, the cells were washed, fresh RPMI medium was added, and the cells were incubated for another 24 hr. Before infection, activated cells were washed again, and fresh RPMI medium was added.

Mycobacterial infection

Twenty-five microliters of MTB isolates at a concentration of $1x10^8$ cells/ml was used to inoculate the activated THP-1 cells at a multiplicity of infection (MOI) of 10. The infection was allowed to take place for 4 hr at 37°C in 5% CO_2. Then, the wells were washed three times with 500 µl PBS to remove extracellular bacteria, and the wells were replenished with 1,000 µl of fresh complete medium. The assays were performed on three different occasions in duplicate, each time with freshly prepared inoculum. The wells with THP-1 cells but lacking bacterial inoculum were used as uninfected controls. The viability of the THP-1 cells was checked with a trypan blue dye exclusion test at all time points (day 0 - day 6). The MTB isolates were inoculated on blood agar plates to ensure that there was no contamination from other bacteria.

Quantitation of intracellular bacterial growth

To monitor intracellular bacterial growth, qPCR was used. The supernatants from each well were removed carefully and stored at -70°C for further cytokine analysis. The intracellular bacteria were released from the cells by adding 250 µl lysis buffer (Cell death ELISA plus®, Roche, Basel, Switzerland) and incubated at room temperature (15ºC to 25ºC) for 30 min. To perform the apoptosis assay, 150 µl of lysate was transferred into new 1.5-ml tubes. The remaining lysate (100 µl) was added to 300 µl of 0.133% Triton X-100 buffer. From each well, 50 µl of lysate was then transferred into 1.5-ml tubes containing 150 µl TE buffer and glass beads in suspension and stored at -70°C for later characterization by qPCR. Following incubation at 80°C for 30 min, the bacterial DNA was extracted with a Hybaid Ribolyser Cell Disrupter at speed 5 for 3 min. The extracted DNA was used as the template for qPCR as described above.

Cell death detection assays

The extent of cell necrosis and apoptosis was detected in infected THP-1 cells at day 1 and day 3 post-infection. Cell death detection ELISA plus® (Roche, Basel, Switzerland) was used to detect mono- and oligonucleosomes in the cytoplasmic fraction of cell lysates (apoptosis) and the supernatant (necrosis). Briefly, 150 µl of cell lysate and supernatant was transferred into 1.5-ml tube containing 150 µl of lysis buffer and centrifuged at 200x g for 10 minutes. For the assays, 20 µl of supernatant (also kit controls) was transferred into a streptavidin-coated microplate. The immuno-reagent (80 µl) was added to the wells and incubated at room temperature for 2 hr. The solution was removed and each well was rinsed three times with 250-300 µl of the incubation buffer. After the buffer solution was removed, 100 µl ABTS solution was added and the microplate was incubated on a shaker until the color developed. ABTS stop solution (100

µl) was added to the well and absorbance was measured at 405 nm. The specific enrichment of mono- and oligonulceosomes released into the cytoplasm was calculated from these values using the following formula: Enrichment factor = Absorbance of the sample/ Absorbance of the uninfected control

Assays for assessing the production of cytokines.

IL-6 and IL-10 production was quantified in the infected THP-1 cells at days 0, 1, 3, and 6 post-infection. TNF-α production in the infected and control THP-1 cells was determined at days 0, 1, 2, and 3 post-infection. Lipopolysaccharide (LPS) was used as a stimulus for the positive control at a concentration of 25 µg/well. The wells of the uninfected cells were used as negative controls. The supernatant from each day was transferred to the microplate and the amount of cytokines was measured by human IL-6, IL-10, and TNF-α ELISA Ready-Set-Go® kit (E-Bioscience, California, USA). The assays were performed in duplicate in all three independent experiments.

Data analysis

The results were presented as the mean ± SD of three independent experiments. Significant differences between the MTB strains were analyzed by Student's *t*-test. The correlation between assay values (intracellular growth, apoptosis, necrosis, and cytokine production) was analyzed by Pearson's test. Statistical analyses were performed using the SPSS software program, version 16.0 (SPSS, Inc.). *P*-values less than 0.05 were considered significant.

Results

Intracellular growth rate of MTB Beijing sublineages.

The intracellular growth rate between five modern and three ancestral MTB Beijing strain in the macrophage cells was compared. The absolute number of bacteria at the starting point and at 4 hrs (day 0) post-infection was 29 bacteria copies/cell and 8.6 bacteria copies/cell, respectively. These copy numbers were comparable among all strains of MTB. The average growth rate was defined as the difference in the number of bacteria on day 1 and day 3. The average growth rate of the ancestral sublineages was significantly higher than that for the modern sublineages (p = 0.013) (Figure 1a). However, their growth rate did not differ significantly from that of the H37Rv control strain. Bacterial growth was not observed when the heat-killed H37Rv strain inoculum was used.

Growth rate of MTB Beijing sublineages in liquid medium.

To determine the growth rate in liquid medium, the MTB strains were inoculated in M7H9 broth and incubated for 1, 3, and 6 days. All samples except the heat-killed H37Rv inoculum showed an increase in the concentration of bacteria in the liquid medium from day 1 to day 6. The growth rate was determined by measuring the difference in OD 600 nm on day 1 and day 6. The ancestral Beijing sublineages exhibited a significantly higher growth rate in liquid medium than the modern sublineages (p = 0.018) (Figure 1b).

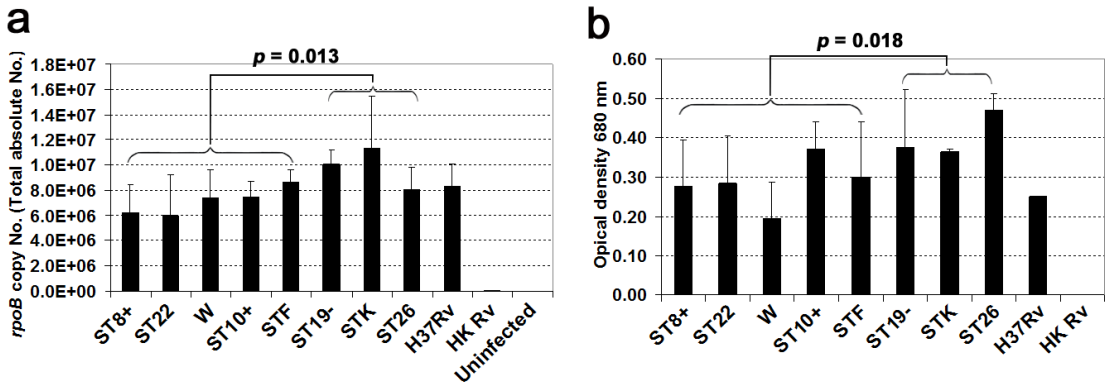

Figure 1. Comparison of intracellular growth (a) and growth in liquid medium (b) in Beijing sublineages (day 3 - day 1).

Note: The modern sublineages are ST8+, ST22, W, ST10+ and STF and the ancestral sublineages are ST19-, STK and ST26. D = day. Intracellular growth was performed by using MOI = 10 and determined by quantitative real-time PCR. The experiments were performed in duplicate on 3 independent occasions. All growth rates were determined at 4 hrs, 24 hrs, 2 days, and 3 days post-infection. The subtracted values of day 3 - day 1 are presented in the graph. The bars represent the averages with standard deviations.

Cytopathic effects of MTB Beijing sublineages.

The average apoptosis level of THP-1 cells induced by infection with the modern Beijing sublineages was significantly lower than that induced by the ancestral sublineages on both day 1 (p = 0.002) and day 3 (p = 0.024) post-infection (Figure 2a). Among 8 Beijing sequence types, only ST8+ and ST22 showed a significantly lower level of apoptosis induction compared to the H37Rv control ($p < 0.05$). The apoptosis level of cells infected with the modern Beijing sublineages on day 1 postinfection was lower than that of uninfected cells (Figure 2a).

On day 1 post-infection, the necrosis level was comparable among the uninfected THP-1 cells and those infected with the Beijing sublineages or the H37Rv control strain. There was no significant difference in the average necrosis level at day 1 between the ancestral and modern Beijing sublineages (p = 0.902). On day 3, the level of necrosis dramatically increased in the ST19-infected cells and STK-infected cells, both of which are the ancestral type. The average necrosis level of the ancestral Beijing sublineages was significantly higher than that of the modern ones (p = 0.003) (Figure 2b). In addition, the intracellular growth rate was directly correlated to the degree of apoptosis induction on day 1 (p = 0.036) and necrosis induction on day 3 (p = 0.001)

IL-6, IL-10, and TNF-α production in macrophages infected by Beijing sublineages.

IL-6 production in cells infected with the modern Beijing sublineages was significantly lower than that for those infected with the ancestral sublineages at all time points examined ($p < 0.05$) (Figure 3a). The amount of TNF-α induced by the modern Beijing sublineages was significantly lower than that produced by the ancestral sublineages but only on day 3 post-infection (p = 0.014) (Figure 3b). At day 3 post-infection, all strains except the ST19 sequence type induced significantly ($p < 0.014$) lower levels of TNF-α than that observed in the H37Rv control (Figure 3b).

MTB infection induced an increase in IL-10 production starting at 4 hrs post-infection and continuing until day 6; the IL-10 level peaked at day 6 post-infection. There were no significant differences in the concentration of IL-10 between macrophage cells activated by infection with the ancestral and modern Beijing sublineages at all time points (Figure 3c).

The correlation analysis revealed a direct correlation of the intracellular growth rate with IL-6 production on day 3 and with TNF-α production on day 1 and an inverse correlation with IL-10 production on day 6 ($p < 0.05$). Significant direct correlations between apoptosis on day 1 and TNF-α on day 2 and on day 3 were found. A significant direct correlation was also observed between apoptosis on day 3 and TNF-α on day 1, on day 2, and on day 3 ($p < 0.05$).

Discussion

The MTB Beijing strain consists of a large number of genetic variants. Several studies on the phenotypic variation within this strain have been reported.[12,13,17,18] However, the underlying virulence factor(s) and the mechanism(s) that impart drug

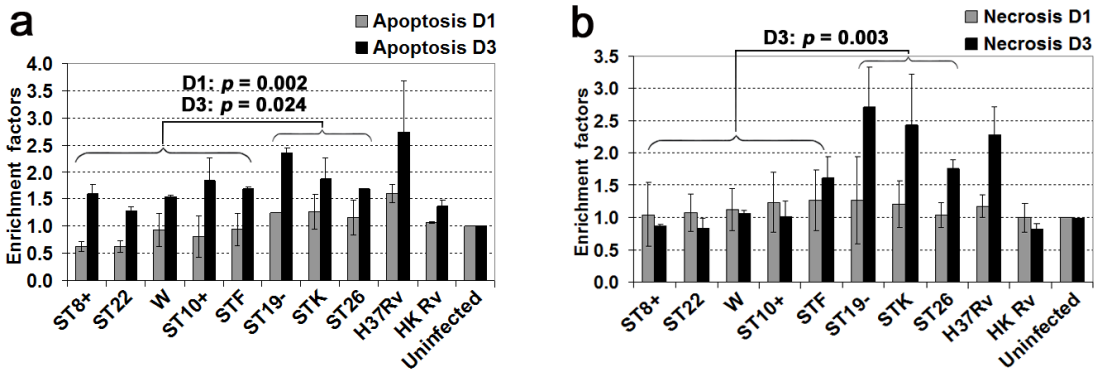

Figure 2. Apoptosis (a) and necrosis (b) in activated macrophages infected with Beijing sublineages.
Note: The modern sublineages are ST8+, ST22, W, ST10+ and STF and the ancestral sublineages are ST19-, STK and ST26. D = day. The experiments were performed in duplicate on 3 independent occasions. The bars represent the averages with standard deviations. Apoptosis and necrosis were measured by detection of nucleosomes in the cytoplasmic fraction of cell lysates and the supernatant, respectively.

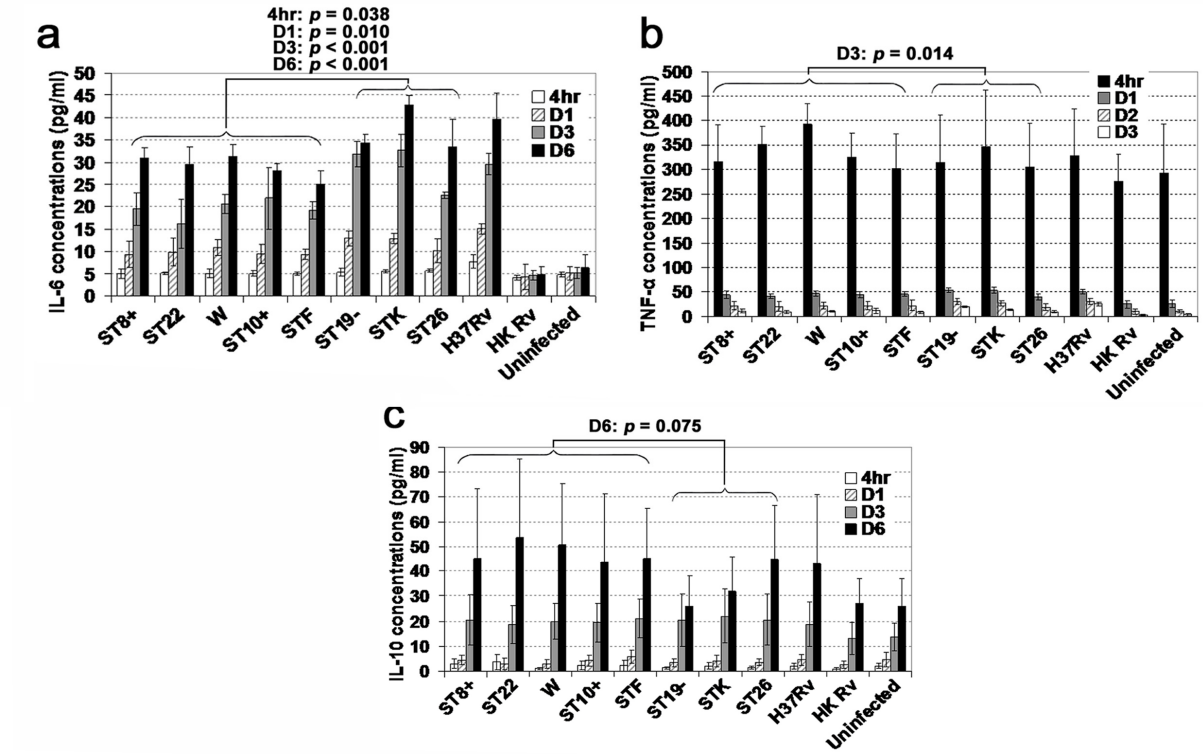

Figure 3. IL-6 (a), TNF-α (b) and IL-10 (c) production by THP-1 infected with Beijing sublineages.
Note: LPS positive control activated the production of IL-6 at the concentrations 55, 236 and 236 pg/ml at days 1, 3 and 6, respectively (not shown on the graph). Modern sublineages: ST8+, ST22, W, ST10+ and STF, ancestral sublineages: ST19-, STK and ST26. D = day. The experiments were performed in duplicate on 3 independent occasions. The bars represent the averages with standard deviations.

resistance have not been clearly identified. In this study, the 8 strains of Beijing MTB were selected to perform phenotypic assays based on the phylogenetic groups from a previous study.[10] These strains were selected to cover all distinct branches from the phylogeny, including the two major subgroups (ancestral and modern). We found that there is a phenotypic variation that is related to the major subgroups of the Beijing strain of MTB.

Previous studies have reported that MTB Beijing sublineages have a wide spectrum of intracellular growth rates in THP-1 macrophages and the growth rate is inversely correlated with TNF-α production.[17-20] Our results confirmed the variation in the intracellular growth rates of the MTB Beijing sublineages in THP-1 macrophage cells. A correlation between intracellular growth and TNF-α production at day 1 post-infection was also noted in this study.

Inhibition of apoptosis is thought to be a means of survival for pathogenic mycobacteria.[21-23] In this study, the modern Beijing sublineages of MTB (ST8+, 22, W, 10+ and F) induced significantly lower apoptosis levels, compared to the ancestral sublineages (ST19-, K, 26). Interestingly, ST8+ and ST22, which are the most recently evolved sequence types,[10] showed the lowest level of apoptosis on day 1 and the level of induction was even lower than in the uninfected control (normal apoptosis level). It has been reported that virulent MTB causes low levels of apoptosis and high levels of necrosis.[23, 24] By contrast, our results show that both apoptosis and necrosis are induced by the modern Beijing sublineages. The mechanisms by which these cytopathic effects are exerted by the MTB Beijing genotype may be different from that of other MTB genotypes or other species of mycobacteria.

IL-6, IL-10, and TNF-α are the key cytokines secreted by infected macrophages. TNF-α and IL-6 have been suggested to play important roles in controlling of MTB infection,[25,26] whereas IL-10 promotes infection.[27] In this study, we found that the ancestral Beijing strain induced lower IL-6 and

TNF-α production. Although insignificant, IL-10 production induced by the ancestral Beijing sublineage was lower than that produced by the modern Beijing sublineage, especially at day 6 post infection. These results might indicate that the ancestral Beijing sublineage induces more production of protective cytokines, TNF-α and IL-6, but induces less nonproductive cytokine, IL-10, compared to the modern Beijing sublineage. Regarding the variations, the SD of the cytokine levels varied at some time points only. For instance, TNF-α at day 3 shows only slight variation. Additionally, statistical analysis of variance of cytokine levels between the two groups ensured the validity of the conclusion.

Recently the correlation between subgroups of Beijing strains and phenotypic properties has been determined.[12,17,18] Lasunskaia et al. compared Russian MDR-highly epidemic strains and Brazilian sporadic strains and found that Russian strains have a higher growth rate, a higher capacity to induce non-protective cytokine synthesis and induce necrosis to a higher level than the Brazilian sporadic strain.[18] Recently, Alonso et al. found that there was no correlation between the intracellular growth rate and the RDs genotype-based phylogeny of MTB in the Mediterranean area.[17] Additionally, Aguilar et al. did not observe a correlation between the phylogeny of MTB Beijing strain sublineages and *in vivo* transmissibility in a mouse model.[28] The present results show a significant variation in virulence which was related to the phenotypes of the Beijing sublineages, associated with their phylogeny. This variation may be due, at least in part, to the higher diversity of the Beijing strain sublineages in our experiment.

Rapid growth rate and the ability to induce necrosis in host cells have been suggested to be associated with the virulence of MTB strains. [20, 29, 30] The virulent H37Rv strain has a faster growth rate than the avirulent H37Ra strain.[31] In the present study, we found that the ancestral Beijing sublineage had a faster growth rate in human macrophages, suggesting that the ancestral Beijing sublineage may have a greater ability to survive as compared with the modern Beijing strain. In the present study, the modern Beijing sublineage caused lower levels of apoptosis and TNF-α and IL-6 production but higher IL-10 production. Previous studies have suggested that the modern Beijing sublineage has higher transmissibility, associated with drug resistance, than the ancestral Beijing strains.[13,32,33] Thus, the modern Beijing sublineage has evolved to have an enhanced ability to suppress host defense mechanisms. However, the slower growth rate and reduced induction of necrosis are beneficial for the maintenance of persistent infection. The higher transmissibility of modern the Beijing sublineage of MTB, as compared to the ancestral sublineage, can possibly be explained by the results of the present study.[33] In contrast, the slower growth rate may be the result of reduced viability *rpoB* was used for growth monitoring of MTB because this gene constitutively expresses the β-subunit of RNA polymerase and the copy number increases when the bacteria multiply. The validation of the qPCR was carried out using the optimized standard curve which indicated that varying the copy number of the *rpoB* gene correlated with the CFU count of MTB (unpublished data).

The genetic differences between the ancestral and modern Beijing sublineages were the IS*6110* in the NTF region and SNPs.[9,10,34] The insertion of IS*6110* in the NTF region is thought to be involved in chromosomal replication of MTB.[1,9,34] and might affect the rate of both intra- and extracellular growth. In addition, non-synonymous SNPs of the putative mutator genes (DNA repair enzymes), are assumed to be involved in the adaptability of the Beijing strain to hostile environments. The ability of the modern Beijing sublineage to induce lower apoptosis and the production of protective cytokines might be explained by these mechanisms.

The limitations of this study lie in the Beijing strains that we selected to perform our experiments. As these Beijing sublineages were originally isolated from TBM patients, there might be some variation within the sublineages due to the selection of meningitis patients only. Nevertheless, the genetic characteristics, IS*6110*, RDs, VNTR 28 loci, SNP 10 loci (with the exception of VNTR 2163, unpublished data), of the Beijing strains in pulmonary TB and meningitis patient groups were comparable between the two disease types. Furthermore, genotype comparison based on IS*6110* patterns between MTB strains isolated from the cerebrospinal fluid and sputum showed no significant differences.[35] Therefore, disease-specific deviation of virulence of the Beijing sublineages is less likely to occur. Secondly, selecting the eight Beijing sequence types found in Thailand for phenotypic comparisons may limit the applicability of the conclusions globally. Due to budget constraints and the experimental work load of the

study, a representative strain from each phylogroup was selected. To our knowledge, this is the largest population of Beijing strains of MTB that have been tested for phenotypic characteristics.

Regarding the variations in the test results, the variation within the modern and ancestral subgroups was in a range that provided statistically significant differences, i.e., the tendency of phenotypic properties between ancestral and modern Beijing MTB was different. The most recently evolved subgroups (ST8+ and ST22) were the strains that induce the least Th1 cytokines and apoptosis. Conversely, ST19 and STK are the 2 strains in the ancestral subgroups that induced the highest Th1 cytokines and apoptosis. The growth rate (intra- and extracellular growth) were also obviously different. The overall differences between all strains in the modern and ancestral subgroups were tested. Even then, the statistical analysis that covers all variations is still significant.

In summary, our results suggest that variation in the phenotypic properties of MTB Beijing strain sublineages is associated with phylogeny. The ancestral Beijing sublineage has a high intracellular growth rate and the ability to induce necrosis and Th1 cytokine production. By contrast, the modern Beijing sublineage induces less apoptosis and protective immune responses than the ancestral Beijing sublineage. This is the first study to demonstrate the correlation between the heterogeneity of the virulence related phenotypes of the Beijing sublineages and molecular phylogenetic groups. The findings of the present study may help to determine the mechanisms or the genetic markers involved in the higher virulence of this genotype and its sublineages.

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